REMARKS/ARGUMENTS

In response to the Office Action of May 17, 2005, Applicants request re-examination and reconsideration of this application for patent pursuant to 35 U.S.C. 132.

Status of Prosecution

Applicants note that the Examiner has checked box 2b of the Office Action Summary form for the action mailed on May 17, 2005, thus indicating that the action is to be considered "non-final". However, at page 18, item 11, the Examiner indicates that the action is to be considered "final". Applicants respectfully request that the Examiner clarify the status of prosecution in the instant application.

Claim Status/Support for Amendments

Claims 36 and 37 have been amended. Claims 2-35 were cancelled in a previous response(filed on June 16, 2003). Claims 1 and 41-43 are withdrawn from consideration. It is understood that claims 1 and 41-43, drawn to a non-elected invention, will remain pending, albeit withdrawn from prosecution on the merits at this time. Claims 1 and 36-43 remain pending in the instant application. Claims 36-40 are under examination.

No new matter has been added by the amendments to the specification made herein.

In the "Background of the Invention" section a punctuation error was corrected at page 1, line 21.

The "Description of the Figures" section has been amended for consistency of language in the figure descriptions.

The paragraph at page 21, beginning at line 7, has been amended to correct typographical errors.

The protocol at page 21, beginning at line 12, has been amended to correct typographical errors and to properly identify trademark names by capitalization.

The paragraph at page 22, beginning at line 2, has been amended to properly identify trademark names by capitalization.

The protocol at page 24, beginning at line 1, has been amended to correct typographical errors, punctuation errors, and to properly identify trademark names by capitalization.

The paragraph at page 24, beginning at line 15, has been amended to properly identify trademark names by capitalization.

The paragraph at page 27, beginning at line 6, has been amended to identify the name AMICON by capitalization. It is uncertain whether AMICON is a trademark or the name of a corporation since it has been cited as both.

In the "Detailed Description" section, the term "cerebrospinal fluid" has been added to define the abbreviation "CSF" at page 28,

line 17 in order to provide additional support for cerebrospinal fluid as recited in claim 38. "CSF" is a well known abbreviation for cerebrospinal fluid in the biochemical art. Assays for determining the presence of the claimed biopolymer markers are discussed at page 31, line 13 to page 33, line 2; spinal fluid is noted to be one type of sample which can be used in the discussed kits. A typographical error within the same paragraph has also been amended (skill replaced skilled).

No new matter has been added by the amendments to the claims made herein.

Claim 36 has been amended to clearly disclose the relationship between the presence of the claimed biopolymer marker (SEQ ID NO:1) and Type II diabetes. Claim 36 has also been amended to explicitly indicate how the presence of the claimed biopolymer marker is determined from mass spectrum profiles. The changes to claim 36 find basis throughout the specification as originally filed, see, for example, page 11, lines 10-14, page 17, lines 11-14, page 27, line 17 to page 28, line 2 and Figures 1 and 2.

Claim 37 has been amended to provide proper antecedent basis for the term "sample".

Objection to the Specification

The Examiner objects to the specification as it allegedly fails to provide proper antecedent basis for the claimed subject

matter; "wherein the presence of an isolated biopolymer marker having SEQ ID NO:1 is indicative of a link to Type II diabetes."

Applicants respectfully disagree with the Examiner's assertion.

According to MPEP 608.01(o), the meaning of every term used in any of the claims should be apparent from the descriptive portion of the specification with clear disclosure as to its import. Furthermore, the terms and phrases used in the claims should find clear support or antecedent basis in the description so that the meaning of the terms in the claims may be ascertainable by reference to the description (37 CFR 1.75(d)(1); see also MPEP 608.01(o)).

At page 27, line 17 to page 28, line 2, SEQ ID NO:1 is identified as indicative of an individual suffering from Type II diabetes. The table in Figure 1 provides evidence to indicate that SEQ ID NO:1 was found in samples obtained from patients having Type II diabetes; thus, SEQ ID NO:1 is considered indicative of Type II diabetes.

The term "link" is used in the instant specification according to its usual meaning, which is, providing a connection or an association between elements. An objective of the instant invention is the evaluation of samples containing a plurality of biopolymers for the presence of disease specific marker sequences which evidence a link to at least one specific disease state (see page

17, lines 11-14).

Therefore, the presence of SEQ ID NO:1 in a sample evidences a link or a connection to Type II diabetes.

In accordance with 37 CFR 1.75(d)(1) Applicants show that the meaning of the phrase, "indicative of a link to Type II diabetes" may be ascertainable by reference to the description and thus respectfully request that the objection to the specification now be withdrawn.

Rejections under 35 USC 112, second paragraph

Claims 36-40, as presented on November 29, 2004, stand rejected under 35 USC 112, second paragraph, as being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Applicants respectfully disagree with the Examiner's assertions.

The Examiner asserts that in claim 36, the preamble is vague and indefinite in relation to step a) in reciting "determining the presence of an isolated biopolymer marker having SEQ ID NO:1" because it is unclear how a biopolymer marker can be present in isolated form and obtained in isolated form from a patient.

Claim 36 has been amended herein to remove the term "isolated".

The Examiner asserts that in claim 36 step b) is ambiguous in

reciting "conducting mass spectrometric analysis on ...peptide fragments obtained therein" because it is unclear how individual peptide fragments are obtained from a patient sample since proteins or biopolymer markers are manifested as full length proteins in native form.

Contrary to the Examiner's assertion, protein fragments have been found in serum, for example, Capiaumont et al. (Clinica Chimica Acta 293:89-103 2000) disclose the finding of HWESAS, a fragment of complement C3, in serum. Furthermore, the digestion of protein samples prior to mass spectrometric analysis is well-known in the art. However, in the interest of compact and efficient prosecution, claim 36 has been amended herein to remove the terms "peptide" and "peptides".

The Examiner alleges that in claim 36, step b) is confusing and lacks clear antecedent support in reciting, "and comparing mass spectrum profiles of said isolated biopolymer marker having SEQ ID NO:1 to mass spectrum profiles of peptide fragments obtained and analyzed from said sample" because it appears that there is only one element (the sample) being compared to itself.

Claim 36 has been amended herein to provide clear antecedent support.

The Examiner asserts that step c) in claim 36 is confusing and lacks clear antecedent support in reciting "confirming the presence of said isolated biopolymer marker having SEQ ID NO:1" because it

is unclear how a biopolymer marker can be present in isolated form and obtained in isolated form from a patient.

Claim 36 has been amended herein to remove the term "isolated".

The Examiner also asserts that it is unclear what element in step b) control sample or patient sample, the recitation of "said isolated biopolymer marker having SEQ ID NO:1" intends to refer back to.

Claim 36, step b) has been amended herein to remove the recitation of the phrase "said isolated biopolymer marker".

Step b) of claim 36 recites "...comparing a mass spectrum profile of a biopolymer marker having SEQ ID NO:1 and a molecular weight of about 1998 daltons to mass spectrum profiles of biopolymers obtained and analyzed from said sample...". The mass spectrum profile of a biopolymer marker having SEQ ID NO:1 is compared to mass spectrum profiles of biopolymers obtained from the test sample in order to determine if there is a match, and, if there is such a match, the test sample contains SEQ ID NO:1.

The Examiner alleges that the last 2 lines in claim 36 are vague and lack clear antecedent support in reciting "the presence of said isolated biopolymer marker" because it is unclear how a biopolymer marker can be present in isolated form and obtained in isolated form from a patient.

Claim 36 has been amended herein to remove the term

"isolated".

The Examiner also asserts that it is unclear what element in steps b) and c) ,control sample or patient sample, the recitation of "said isolated biopolymer marker having SEQ ID NO:1" intends to refer back to.

Claim 36, step b) has been amended herein to remove the recitation of the phrase "said isolated biopolymer marker".

In step c) it is clear that the phrase "said biopolymer marker" refers back to the biopolymer marker identified in the patient sample, if such a biopolymer marker is found to be present.

Accordingly, Applicants have now addressed the Examiner's concerns under 35 USC 112, second paragraph and have clarified the metes and bounds of the claims. Thus, Applicants respectfully request that these rejections under 35 USC 112, second paragraph be withdrawn.

Rejection under 35 USC 112, first paragraph (New Matter)

Claims 36-40, as presented on November 29, 2004, stand rejected under 35 USC 112, first paragraph, as failing to comply with the written description requirement as the claims allegedly contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

The Examiner alleges that the specification does not appear to provide literal or adequate descriptive support for the recitation of "biopolymer marker having SEQ ID NO:1 is indicative of a link to Type II diabetes". Applicants' disclosure only provides that the biopolymer marker having SEQ ID NO:1 is indicative of an individual suffering from Type II diabetes, but provides no specific showing of its specific link to the disease, how it is linked to the disease, or what aspect of the disease the marker is intended to be linked to. Additionally, none of the originally filed claims recited the limitation in question.

Applicants respectfully disagree with the Examiner's contention and assert that the recitation of "biopolymer marker having SEQ ID NO:1 is indicative of a link to Type II diabetes" is supported in the application as originally filed.

The Examiner is reminded that amendments to an application which are supported in the original disclosure are NOT new matter (see MPEP 2163.07).

A biopolymer marker having SEQ ID NO:1 is disclosed in the specification as originally filed (see page 27); the term "indicative" is used in the specification as originally filed (page 28) and the term "link" is used in the specification as originally filed (see page 17).

At page 27, line 17 to page 28, line 2, SEQ ID NO:1 is identified as indicative of an individual suffering from Type II

diabetes. The table in Figure 1 provides evidence to indicate that SEQ ID NO:1 was found in samples obtained from patients having Type II diabetes; thus, SEQ ID NO:1 is shown to be indicative of Type II diabetes.

The term "link" is used in the instant specification according to its usual meaning, which is, providing a connection or an association between elements. An objective of the instant invention is the evaluation of samples containing a plurality of biopolymers for the presence of disease specific marker sequences which evidence a link to at least one specific disease state (see page 17, lines 11-14).

Therefore, the presence of SEQ ID NO:1 in a sample evidences a link or a connection to Type II diabetes.

Applicants recitation of the phrase "biopolymer marker having SEQ ID NO:1 is indicative of a link to Type II diabetes" is a mere rephrasing of terms which were disclosed in the specification as originally filed. Such rephrasing is permitted (see MPEP 2163.07 I).

Accordingly, Applicants have now shown that the alleged new matter has support in the specification as originally filed and thus, should not be considered "new matter". Applicants respectfully request that this rejection under 35 USC 112, first paragraph be withdrawn.

Rejection under 35 USC 112, first paragraph (enablement)

Claims 36-40, as presented on November 29, 2004, remain rejected under 35 USC 112, first paragraph, as containing subject matter which allegedly was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The Examiner maintains that the recitation of "presence of a biopolymer marker having SEQ ID NO:1 is indicative of a link to Type II diabetes" is not adequately supported by the specification because there is no basis for how and why a biopolymer marker having SEQ ID NO:1 and molecular weight of 1998 should be linked to Type II diabetes. Nowhere in the specification provides adequate description and data to support the assertion that a biopolymer marker having SEQ ID NO:1 is specifically indicative of a link to Type II diabetes. There is no evidentiary showing, given the instant specification and Figures 1 and 2, that one skilled in the art would have deduced that the claimed biopolymer marker is a reactive marker that provides indication of a link to Type II diabetes, because a population of 7 subjects is not a significant assay pool to draw such a conclusion. The Examiner further maintains that nowhere in the disclosure has shown the nexus between the claimed biopolymer marker and its link to Type II diabetes.

Applicants respectfully disagree with the Examiner's assertions.

The case law has established that a "test of enablement" is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the prior art without undue experimentation (see MPEP 2164.01).

Furthermore, the decision in *In re Brandstadter* (179 USPQ 286; MPEP 2164.05) has established that the evidence provided by applicant (to overcome an enablement rejection) need not be conclusive but merely convincing to one of skill in the art.

Claim 36 has been amended herein to clarify that the presence of the biopolymer marker (SEQ ID NO:1) is indicative of Type II diabetes. Applicants respectfully submit that the instant specification provides sufficient evidence to convince one of skill in the art that the biopolymer marker(SEQ ID NO:1) is indicative of Type II diabetes.

At page 27, line 17 to page 28, line 2, SEQ ID NO:1 is identified as indicative of an individual suffering from Type II diabetes. The table of data in Figure 1 provides evidence to indicate that SEQ ID NO:1 was found in samples obtained from patients having Type II diabetes. The figure attached to the Declaration (under 37 CFR 1.132) filed on June 16, 2003 provides side-by-side mass spectrometric profiles, the top profile obtained

from a patient having Type II diabetes and the bottom profile obtained from a patient determined to be normal with regard to Type II diabetes. This profile comparison clearly evidences the lack of the 1998 dalton biopolymer marker (SEQ ID NO:1) in a sample obtained from a normal patient (healthy control). Thus, the appearance of the characteristic profile of SEQ ID NO:1 in a sample is considered to be indicative of Type II diabetes.

In accordance with the claimed method, the mass spectrometric profile of SEQ ID NO:1, as shown in Figure 2, is used as a reference for comparison with mass spectrometric profiles of peptides obtained from unknown test samples.

Mass spectrometry is common practice in proteomics and Applicants respectfully submit that one of skill in this art would know how to carry out mass spectrometry protocols and interpret data obtained from such protocols; especially when the objective of one's research is the identification of biomarkers of particular physiological states.

For example, Scott D. Patterson presents the state of the art in mass spectrometry/proteomics (in 2000) by summarizing the Asilomar Conference on Mass Spectrometry (see attached article, Physiological Genomics 2:59-65 2000; reference 1). This conference took place in 2000, thus coinciding with the time that the instant inventors were working to develop the instant invention.

For example, at page 64, left column of Patterson (see attached article, Physiological Genomics 2:59-65 2000; reference 1) is a description of the SELDI approach (as discussed at the conference by Scot Weinberger) wherein defined chemical/biochemical surfaces are utilized to allow fractionation of proteins from biological fluids in a reproducible manner. This reproducibility allows comparisons between different samples to be made. Weinberger also described a search for markers of benign prostate hyperplasia that, like prostate cancer, displays elevated prostate specific antigen (PSA) levels. The fraction exhibiting a difference between these samples was able to be enzymatically digested, and a number of peptides were generated. These peptides were able to be fragmented using the MALDI-Qq-TOF(a procedure described by Ken Standing at the conference, page 62, left column of Patterson). It was found that there appears to be a difference in the relative level of seminogelin fragments between these two states (prostate cancer and benign prostatic hyperplasia), thus providing a potential differential marker.

Applicants respectfully draw the Examiner's attention to the fact that the method described by Weinberger is analogous to the method described in the instant specification; the exception being the preparatory steps of the instant invention. Furthermore, when interpreting data Weinberger uses the same approach to interpretation as the instant inventors in order to identify

seminogelin fragments as a potential marker to distinguish between benign prostate hyperplasia and prostate cancer based on differential expression of the fragments. Applicants also respectfully point out to the Examiner that the only criteria used by Weinberger to identify seminogelin as a potential marker was the difference in relative level of a seminogelin fragment found between the two disease states.

Additionally, Hutchens et al., in US Patent 6,225,047, disclose that one of the goals of proteomics is the identification and characterization of organic biomolecules that are differentially expressed between cell types. Hutchens et al. note that by comparing expression one can identify molecules that may be responsible for a particular pathogenic activity of a cell. For example, identifying a protein that is expressed in cancer cells but not in normal cells is useful for diagnosis, and ultimately, for drug discovery and treatment of the pathology (see attached relevant pages of Hutchens et al. column 1, lines 37-45; reference 2).

It can be seen from these examples taken from the prior art that identifying potential biopolymer markers based upon their differential expression between a disease state and a normal physiological state is an acceptable practice. Thus, with reference to the claimed method, the appearance of the characteristic profile of SEQ ID NO:1 in a sample would be enough to convince one of skill

in the art that SEQ ID NO:1 is indicative of Type II diabetes. Accordingly, the instant invention is in compliance with the criteria as set forth in *In re Brandstadter* .

Furthermore, Applicants assert that those of skill in the art are both highly knowledgeable and skilled and it is obvious that no undue experimentation would be required for a skilled artisan to follow any of the chromatographic and mass spectrometric protocols presented in the instant specification in order to use the claimed invention. It is known that mass spectrometry submits proteins to degradation, generating a re-producible pattern of sequence-defining fragments, thus, mass spectrometric profiles can be used as reference points for identification of proteins present in an unknown sample. Accordingly, one of skill in the art would be able to ascertain whether the known peptide (SEQ ID NO:1) is present in an unknown sample by comparing the mass spectrometric profile of SEQ ID NO:1, as shown in Figure 2, with mass spectrometric profiles obtained from an unknown sample.

The Examiner asserts that the instant specification does not contain working examples, however, Figures 1 and 2 present data, which clearly evidences the presence of working examples. This data discloses that the peptide (SEQ ID NO:1) was identified in samples obtained from Type II diabetes patients, but was not identified in samples obtained from normal patients, thus it can be reasonably predicted that SEQ ID NO:1 is indicative of Type II diabetes.

Thus, Applicants contend that a skilled practitioner would find that the data presented in the instant specification is convincing. Furthermore, considering the above comments, it is clear that both the specification and the prior art disclose how to make and use the invention. Accordingly, Applicants respectfully contend that the instant invention satisfies the "test for enablement" since one skilled in the art could make or use the invention from the disclosures in the specification coupled with information known in the prior art without undue experimentation.

The Examiner asserts that there is no evidentiary showing, given the instant specification and Figures 1 and 2, that one skilled in the art would have deduced that the claimed biopolymer marker is a reactive marker that provides indication of a link to Type II diabetes, because a population of 7 subjects is not a significant assay pool to draw such a conclusion.

However, previously in the art, conclusions, similar to those of the instant inventors, have been made using study groups with a small number of participants. For example, Capiaumont et al. (Clinica Chimica Acts 293:89-103 2000; cited by the Examiner) use groups with small numbers of participants, see Table 1. Capiaumont et al. conclude from their preliminary results that the fragment HWESAS could be a marker of renal function and a prognostic factor in renal transplantation using data obtained from experiments with these small study groups (see page 101). Thus, considering that

small assay pools have been successfully applied previously, the Examiner's assertions regarding the size of the assay pool, are not sufficient to challenge the validity of the association between SEQ ID NO:1 and Type II diabetes disclosed in the instant specification.

The Examiner asserts that the prior art of record fails to disclose a method for determining the presence of a biopolymer marker having SEQ ID NO:1 in a patient sample wherein the presence of the biopolymer marker having SEQ ID NO:1 is indicative of a link to Type II diabetes. Thus, the Examiner asserts that the state of the prior art is unpredictable. Apparently, the Examiner believes that because the art does not disclose that SEQ ID NO:1 is indicative of Type II diabetes, SEQ ID NO:1 can not be indicative of Type II diabetes.

Applicants respectfully point out to the Examiner that the fact that the prior art does not disclose complement C3f as a marker for Type II diabetes does not automatically render the instant invention "unenabled" since it has been established that the mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it (see MPEP 2164.02).

The table shown in Figure 1 evidences that SEQ ID NO:1 was identified in samples obtained from patients having Type II

diabetes and thus, supports the use of SEQ ID NO:1 in detection of Type II diabetes.

The guidelines for a "test of enablement" indicate that if a statement of utility in the specification contains within it a connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated, 35 USC 112, is satisfied (see MPEP 2164.01(c)).

Although the prior art does not specifically recognize that SEQ ID NO:1, a fragment of complement C3f, is related to Type II diabetes, it does recognize that elevated levels of C3 have been reported in Type II diabetes (see attached abstract of Weyer et al. Diabetes Care 23(6):779-785 2000; reference 3). Considering the information presented in the instant specification and the information in the prior art regarding the increased levels of C3 found in Type II diabetes, Applicants contend that when one of skill in the art observes that SEQ ID NO:1 was identified in Type II diabetes patients but was not identified in healthy "control" patients; one of skill in the art would connect SEQ ID NO:1 with potential diagnostics and/or therapeutics for Type II diabetes.

Thus, Applicants respectfully assert that since the specification demonstrates that SEQ ID NO:1 is indicative of Type II diabetes and that this indication connotes the use of SEQ ID NO:1 in potential diagnostics and/or therapeutics of Type II diabetes, the requirement of "how to use" under 35 USC 112, first

paragraph is satisfied.

Furthermore, Applicants respectfully submit that one of ordinary skill in the art would find the suggestion of SEQ ID NO:1 as a marker for Type II diabetes to be reasonable.

At page 27, line 17 to page 28, line 2, of the specification as originally filed, SEQ ID NO:1 is identified as a fragment of complement C3f. As mentioned above, elevated levels of C3 have been reported in Type II diabetes (see attached abstract of Weyer et al. Diabetes Care 23(6):779-785 2000; reference 3). Additionally, it has also been shown that activation of the complement system may be play a role in the development of macrovascular disease in Type II diabetes (see attached abstract of Figueredo et al. Diabetes Care 16(2):445-449 1993; reference 4). One of skill in the art, considering that the complement system is activated in Type II diabetes and levels of complement C3 are elevated in Type II diabetes, upon observation of the presence of SEQ ID NO:1 in samples obtained from patients having a history of Type II diabetes corresponding with the absence of SEQ ID NO:1 in samples obtained from healthy control patients, would find it reasonable to believe that SEQ ID NO:1 is indicative of Type II diabetes. Therefore, one of ordinary skill in the art would recognize the connection of SEQ ID NO:1, elevated levels of complement C3 and Type II diabetes and thus would also find the suggestion of SEQ ID NO:1 as a marker for Type II diabetes entirely reasonable. In other words, the elevated

levels of complement C3 found in Type II diabetes can be considered a nexus between SEQ ID NO:1 and Type II diabetes.

The Examiner cites an article by Capiaumont et al.(Clinica Chimica Acta 293:89-103 2000) which is deemed relevant to the argument that the instant invention is not enabled.

In the previous Response, filed on November 29, 2004, Applicants noted that the claims do not include or exclude the possibility of renal failure or any other condition in patients in which the peptide (SEQ ID NO:1) is positively identified.

The Examiner asserts that Applicants' argument is not on point because the Capiaumont et al. reference is not relied upon in the context of an art rejection whereupon a teaching within the reference reads on the claimed invention.

Applicants respectfully contend that the Examiner's interpretation of Applicants' argument is incorrect.

Applicants view Capiaumont et al. as a reference alleged to support an argument that the instant invention is not enabled, and do not view such reference as supporting an art rejection.

A reference used in support of an enablement rejection should provide evidence of what one skilled in the art would have known about the problem to be solved on or before the effective filing date of the patent application (see MPEP 2164.05(a)). Although Capiaumont et al. is concerned with complement fragments as markers of disease (renal pathology), they do not disclose anything to

indicate what one of skill in the art would know regarding complement as markers of any pathology other than renal pathology. However, Applicants respectfully submit that the information Capiaumont et al. does disclose about peptide markers of disease conditions actually supports enablement of the instant invention rather than supports an argument for "unenablement" as was intended by the Examiner. Capiaumont et al. measured the serum concentration of the complement fragment HWESAS in groups of both healthy patients and patients having chronic renal failure and found that the concentration of HWESAS decreases or becomes undetectable in patients with chronic renal failure (as compared to concentration in healthy patients) and increases after successful kidney transplantation. Capiaumont et al. determined from these results that the HWESAS peptide could be a marker of renal function.

In the same manner as Capiaumont et al., the instant inventors determined from their findings of differential expression of SEQ ID NO:1 in Type II diabetes patients versus healthy patients that SEQ ID NO:1 could be a marker for Type II diabetes.

Furthermore, Capiaumont et al. is silent with regard to the presence of complement fragments in Type II diabetes, thus, one of skill in the art would not be able to ascertain any knowledge about the identification of SEQ ID NO:1 in Type II diabetes made by the instant inventors from the teachings of Capiaumont et al., for example, Capiaumont et al. do not disclose any doubts about

connecting HWESAS with renal function based on their disclosed data nor do they disclose that complement is an exclusive marker for renal function. In fact, Capiaumont et al. do not teach anything which would cast the teachings of the instant invention regarding SEQ ID NO:1 and Type II diabetes in a dubious light.

Accordingly, based upon the above comments, Applicants respectfully submit that the article of Capiaumont et al. is not sufficient to support the Examiner's rejection for lack of enablement.

The Examiner asserts that Appendix A (filed with a declaration under 37 CFR 1.132 on November 29, 2004) does not appear to provide evidentiary showing that a population of previously unknown subjects can be specifically identified as having an indication of a link to Type II diabetes using the claimed method.

Applicants respectfully submit that an evidentiary showing that a population of previously unknown subjects can be specifically identified as having an indication of a link to Type II diabetes is not necessary to provide evidence for enablement of the instant invention. The serum content of the patients listed in Appendix A was compared with the serum content of healthy patients and the differentially expressed peptides were subjected to chromatographic and mass spectrometric procedures for identification. Mass spectrometric profiles are reproducible and thus were used to formulate a library of proteomic references.

Therefore, the mass spectrometric profile of SEQ ID NO:1 as disclosed herein is used as a reference point for comparison with mass spectrometric profiles of unknown samples. One of ordinary skill in the art would know how to use the mass spectrometric profile of SEQ ID NO:1 (Figure 2) to screen patients for indication of Type II diabetes.

Thus, in contrast to the Examiner's assertion, no evaluation of unknown subjects is necessary to practice the instant invention as claimed.

Furthermore, fragments of complement C3f of various molecular weights were identified from samples obtained from Type II diabetes patients; for example, the peptide of 1777 daltons (see page A8), the peptide of 1865 daltons (see page A9), the peptide of 1998 daltons (SEQ ID NO:1 of the instant invention; see page A11), and the peptide of 2021 daltons (see page A11). The presence of four fragments of complement C3f in Type II diabetes further evidences an association between complement C3f and Type II diabetes. As can be seen from review of Appendix A, the complement C3f fragment having a molecular weight of 1998 daltons (SEQ ID NO:1) was identified exclusively in Type II diabetes (see page A11 especially), thus SEQ ID NO:1 can specifically separate Type II diabetes from other disease conditions listed in Appendix A.

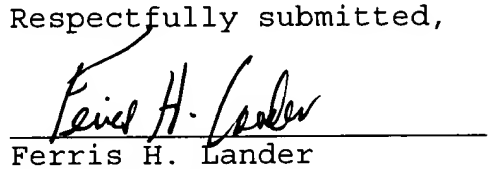
In conclusion, Applicants assert that the presence of SEQ ID NO:1 in Type II diabetes and the corresponding absence of SEQ ID

NO:1 in healthy patients evidences that SEQ ID NO:1 is indicative of Type II diabetes; a statement which is enabled by the instant specification, as evidenced by the arguments presented herein. Applicants assert that one of ordinary skill in the art, when reviewing the instant specification, given the level of knowledge and skill in the art, would recognize the connection between SEQ ID NO:1 and Type II diabetes and would further recognize how to use SEQ ID NO:1 as a marker for Type II diabetes. Thus, Applicants respectfully request that this rejection under 35 USC 112, first paragraph now be withdrawn.

CONCLUSION

In light of the foregoing remarks, amendments to the specification and amendments to the claims, it is respectfully submitted that the Examiner will now find the claims of the application allowable. Favorable reconsideration of the application is courteously requested.

Respectfully submitted,



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Mass spectrometry and proteomics

2132.013
Examiner copy
reference #1

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THE 15TH ASILOMAR CONFERENCE on Mass Spectrometry this October was devoted to the role of mass spectrometry (MS) in proteomics. The Asilomar Conference site is in a picturesque national park in Pacific Grove, CA, overlooking the Pacific Ocean. The conference aims to bring together scientists from a cross section of disciplines that are applying MS to an emerging field. This year, that emerging field is proteomics. The term "proteome" was coined by Wilkins et al. (17) in the mid-1990s to describe the protein complement of the genome. The term was first used to describe the 20-yr-old field of two-dimensional gel electrophoresis (2-DE) and quantitative image analysis. 2-DE remains the highest resolution protein separation method available, but the ability to identify the observed proteins was always an extremely difficult problem. MS has been integral to solving that problem. Although improvements in 2-D gel technology had been realized since its introduction, three enabling technological advances have provided the basis for the foundation of the field of proteomics. The first advance was the introduction of large-scale nucleotide sequencing of both expressed sequence tags (ESTs) and, more recently, genomic DNA. The second was the development of mass spectrometers able to ionize and mass-analyze biological molecules and, more recently, the wide-spread introduction of mass spectrometers capable of data-dependent ion selection for fragmentation (MS/MS) (i.e., without the need for user intervention). The third was the development of computer algorithms able to match uninterpreted (or partially interpreted) MS/MS spectra with translations of the nucleotide sequence databases, thereby tying the first two technological advances together. Thus MS played a key role in the passage of 2-DE/image analysis to proteomics.

As a note to readers unfamiliar with MS, the instruments are named for their type of ionization source and mass analyzer (see also Refs. 1, 11, 12). To measure the mass of molecules, the test material must be charged (hence ionized) and desolvated (dry). The two most successful mechanisms for ionization of peptides and proteins are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In MALDI the analyte of interest is embedded in a matrix that is dried and then volatilized in a vacuum under ultraviolet laser irradiation. This is a relatively efficient process that ablates only a small portion of the analyte with each laser shot. Typically, the mass analyzer coupled with MALDI is a time-of-flight (TOF) mass analyzer that simply measures the elapsed time from acceleration of the charged (ionized) molecules through a field-free drift region. The other common ionization source is ESI, in which the analyte is sprayed from a fine needle at high voltage toward the inlet of the mass spectrometer (which is under vacuum) at a lower voltage. The spray is typically either from a reversed-phase HPLC (RP-HPLC) column or a nanospray device (19) that is similar to a microinjection needle. During this process, the droplets containing analyte are dried and gain charge (ionize). The ions formed during this process are directed into the mass analyzer, which could be either a triple-quadrupole, an ion trap, a Fourier-transform ion cyclotron resonance (FT-ICR), or a hybrid quadrupole TOF (Qq-TOF) type.

This Asilomar meeting provided one of the largest academic forums in the United States for the presentation and discussion of MS as it is applied to proteomics. As is obvious from the introduction, the initial role MS played was as a protein identification and characterization methodology. However, the role of MS is expanding in this field. Although a series of talks focused on the use of different kinds of MS to identify gel-separated proteins and the various automation technologies applied to perform this in high throughput, several talks also presented alternate approaches. These approaches utilized direct analysis of digested protein mixtures for either identification of the components or quantitative analysis of two different samples mixed together. Specific biological applications were also presented. As described above, a critical component of any MS approach as applied to proteomics is the computational analysis. This report will be divided to focus on these six aspects of MS in proteomics. Where references are known for some of the material presented, they are cited. The program was, however, not entirely limited to MS in proteomics. Prior to the six sections covering the conference core, the first section of this report covers those presentations that were aimed at providing an insight into broader biological and drug discovery processes.

Proteomics in biology and drug discovery. The opening lecture, given by Lee Hood (Univ. of Washington), provided an excellent overview of Genomics, Proteomics, and Systems Biology. Hood described the genome project efforts that provide four types of maps: genetic, physical, gene, and sequence. For the human genome,

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it is anticipated that 90–95% of all genes will be sequenced sometime next year. This is the first step toward what Hood described as the "Periodic Table of Life." The different approaches to genomic sequencing and microarray technologies that are able to interrogate the mRNA levels of thousands of genes at a time were described. Hood described proteomics in broad terms as the study of multiplicity of proteins. The information obtained from the various hierarchical levels of biological information (gene, protein, pathways, interconnecting pathways) must be integrated for us to be able to provide a more complete biological picture. For both microarray and proteomics, samples representing the disease process must be obtained. This means that pure cell populations must be microscopically captured from tissues and/or sorted prior to analysis. Therefore, analyses at the mRNA and protein level must be conducted at very low levels and substantial engineering opportunities exist in the biological field to provide the necessary solutions. However, generation of the data is only the first hurdle, as the analysis of the data from a systems perspective then must be undertaken. Hood presented systems biology as the challenge for the 21st century and provided a series of examples of large-scale approaches to biology, from genome sequencing of unicellular organisms, to the sequencing of the T-cell receptor locus, to cancer biology, all of which benefit from such approaches.

Three other presentations were included in the program, to provide a broader background to the utilization of proteomics in drug discovery. Doug Buckley (Exelixis) described the generic view of the drug discovery pipeline, the various "choke" points in the process, and where proteomics could play a role. Of note was the discussion of the changing patent protection landscape, during which Buckley said that full-length cDNA patents were being issued despite the existence of EST patents on portions of these genes. Buckley also predicted that functional data is expected to be required for patents beyond the inferences gained from bioinformatics. The choke points he referred to were target validation, assay development, mechanistic biology, and toxicology. Exelixis is using model organisms (*Caenorhabditis elegans*, *Drosophila*, mouse, and zebrafish) to screen for genes that disrupt/modulate pathways common between man and these organisms. Roles for proteomics included follow-up on targets (direct analysis of protein differences, proteins associated with gene products of interest), assay development [e.g., validation of hits in high-throughput screening (HTS)], and mechanistic biology (e.g, comprehensive analysis of a knockout phenotype). Most importantly, Buckley presented the bottom line that all new technologies must demonstrate their worth by concrete changes in the drug development pipeline (i.e., greater efficiency, better decisions). He predicted that proteomics could provide these benefits at the multiple restriction points referred to above.

Pharmacoproteomics, using 2-DE to profile mechanisms of drug efficacy and toxicity, was presented by Tina Gatlin (Biosource/Large Scale Biology Corporation). The synergy between mRNA expression profiling (for low-abundance gene products) and protein expression profiling (for posttranslational modifications and subcellular localization) was presented. An exception to this is the search for surrogate markers, where secreted proteins were normally the choice and in which there is no identifiable mRNA source to mirror serum or urine protein expression. The aim of their Molecular Effects Database of 2-DE patterns, obtained from livers of drug-treated rats, is to establish links between expression patterns and toxic endpoints to reveal markers for efficacy and prediction of side effects which can be used for lead selection. In disease models, the hypothesis is that the altered expression pattern could be reversed by treatment with a drug.

The closing presentation of the meeting, given by Jeff Seilhamer (Incyte), presented analyses of the precursor to proteins, mRNA. The staff at Incyte have generated very large EST libraries and from these have estimated the number of genes in the human genome to be 129,769 (based on CpG island estimates, 142,634). They are now sequencing the human genome at a rate of about 1 million reads a month on the Megabace platform with 9 sequencing runs/day. Assembly of the data is being accomplished using Linux on 1,500 CPUs (160 computers) with 75 terabytes of storage capacity. Single-nucleotide polymorphisms (SNPs) are being calculated from their sizable EST collection, and mRNA expression profiling is being achieved using their GEM microarray platform. These data are being integrated with 2-DE proteomics data being generated by their partner Oxford GlycoSciences. This integration of the technologies of genomics and proteomics forms the basis of their drug discovery approach for profiling differences between normal and diseased tissue.

Computational aspects of proteomics. Determining the masses of peptides (MS spectra) derived from enzymatic digestion of gel-separated proteins is often the first step in a mass spectrometric-based protein identification strategy. Peptide-mass mapping is the most commonly employed mass spectrometric approach for protein identification from organisms whose genome is completely sequenced (or at least for which the more abundantly expressed genes have been sequenced). The basis of the method is the matching of experimentally determined peptide masses with peptide masses calculated for each entry in a sequence database (using the specificity of the enzyme used to generate the experimental data). How well the experimentally determined masses match with the calculated masses forms the basis of the approach. Ron Beavis (Proteometrics) described how to obtain high-quality data, which even if less, are better than more low-quality data. The use of specific matrices as well as the use of standards with respect to obtaining appropriate data sets for peptide-mass mapping was addressed. Later in the day David Fenyo (Proteometrics) described how to utilize this data in a three-step process as is performed in their WWW-available program, Profound, which uses a Bayesian algorithm (**http://www.proteometrics.com**). The process is as follows:

1) assignment of monoisotopic masses to the raw data, *2*) peptide-mass search, and *3*) significance testing of the result (4). The last step was presented as the most critical because it is from this that the confidence of the match is derived. This is achieved through calculation of a score frequency function for false positives. This was derived from statistical analysis of the database being searched using random selections of peptide masses from different proteins that are then grouped as synthetic proteins and used in a peptide-mass search of the database in question. This is repeated for a variety of random selections to come up with robust statistics for false positives.

The next level of protein identification is the generation of fragment ion spectra from peptides isolated in the gas phase of the mass spectrometer (MS/MS spectra). Matching of fragment ion spectra follows the same principle as for peptide-mass mapping. Experimentally calculated masses of fragment ions (together with the intact mass of the peptide, and often the specificity of the enzyme used to generate the peptide) are matched with those calculated for isobaric peptides (i.e., same mass as experimentally determined) from entries in sequence databases. Arthur Moseley (Glaxo Wellcome) described how nanoscale capillary LC-MS/MS (where peptides are separated chromatographically before MS/MS) had been automated for identification of gel-separated proteins. The throughput of this 75 μm ID capillary system connected to a Qq-TOF mass spectrometer was 20 samples per day at levels to 30 fmol (loaded on gel) for BSA. Moseley continues to develop ultra-HPLC (in some cases combined with variable flow systems) that improve both the speed and resolution of separation. In a Glaxo Organelle Proteomics program, various approaches to protein identification were examined. A comparison of the total number of proteins identified following in situ enzymatic digestion of proteins separated by either high-resolution 2-DE or one-dimensional (usually SDS-PAGE) gel electrophoresis (1-DE) was presented. Only one or a limited number of proteins are present in each of the 2-DE spots, whereas many proteins were present in the 1-DE bands of the enriched Golgi complex. In fact, more proteins were identified from the 1-DE bands than from the 2-DE spots (see below, *Analysis of complex protein mixtures without gel electrophoresis*).

MS/MS spectra derived from tryptic digestions conducted in the presence of equal quantities of $H_2{}^{16}O$ and $H_2{}^{18}O$, when combined with subtractive analysis of the two types of spectra, allows de novo sequencing as described by Matthias Wilm (EMBL) (18). By utilizing a Qq-TOF mass spectrometer, peptides containing both COOH-terminally incorporated stable isotopes and just the isoform containing the ^{18}O could be selected for fragmentation from the mixture. Subtraction of the ^{18}O spectrum from the ^{16}O:^{18}O spectrum reveals only ^{16}O *y*-series ions. Often, a complete ion series is obtained. The method has proved feasible in their hands when 1 pmol of protein is present in the gel (1/4 of this amount can be successfully analyzed with standard digest conditions).

Automated identification of gel-separated proteins by mass spectrometry. Following quantitative analysis of 2-DE patterns, the next step is the identification of all protein spots that display differential expression. Andrew Gooley (Proteome Systems) described the approaches they are employing for quantitative analysis using 2-DE. This included the following: sample preparation (sequential detergent extraction with aminosulfobetaine-14), narrow-range immobilized pH gradient (IPG) with mini-gels for the 2nd dimension, through to the robotic system that they have codeveloped for spot excision, liquid handling (peptide extraction and reverse-phase bead cleanup and storage) and peptide-mass fingerprinting by MALDI-MS. Apart from the throughput of the robotic system, diminished contamination from keratin and more reproducible spotting of samples for MALDI-MS is a highly desirable feature of automation. Hans-Werner Lahm (Hoffmann-La Roche) described the high-throughput system they use for automated spot excision from 2-DE, digestion (with low-salt buffer to eliminate the need for cleanup), and spotting for automated MALDI-MS. Lahm also described the computational aspects of operating such a system in high-throughput mode for long periods of time, including automated database search routines for users distributed throughout the world at other Roche sites. They are investigating the use of stable isotope labeling ($^{14}N/^{15}N$) followed by mixing of each sample prior to 2-DE for direct quantitation of relative expression differences from the MALDI-MS spectra of individual protein spots. The system averages 1,000 spots to spectra per day (including downtime).

David Arnott (Genentech) described automation of in-gel digestions following analysis of differentially regulated proteins from 2-DE. Arnott described the trapping cartridge approach that was required to analyze extracted peptides from the DigestPro robot (currently 30 sample spots, but upgradeable to 96) by microcapillary LC-MS/MS. They aimed to automate as much of the sample processing as possible with automated liquid handling from the digestion robot to the data-dependent LC-MS/MS (capable of handling 40 samples per day) using an ion-trap mass spectrometer followed by auto-database searching using Sequest (3). The system is capable of analysis of subpicomolar quantities of protein from silver-stained gels.

Advances in separations and mass spectrometers. Accurate mass analysis of intact proteins using an 11.5-T FT-ICR coupled with a capillary electrophoresis (CE) instrument was demonstrated by Richard Smith (Pacific Northwest National Laboratory) as a means of proteome analysis. Through the use of stable isotope labeling of one sample and running that sample with an unlabeled sample provides the possibility to measure protein expression ratios. To identify the proteins that display different ratios, dissociation in the FT-ICR-MS to yield mass tags is possible. Having intact mass information as well as identification allows post-translational modifications to begin to be investigated. The mass accuracy obtainable by this FT-ICR-MS was said to be <0.75 ppm which allows the generation of

accurate mass tags for tryptic peptides. In many cases this may be sufficient for protein identification (at least for an organism like *C. elegans*). In some cases, MS/MS may be required, but once performed it would not have to be repeated. Another possibility is the identification of cysteine-containing peptides at a mass accuracy of 1 ppm, which was said to be sufficient for identification. Another possibility being explored with this instrument is multiplexed MS/MS, where up to 7 ions could be isolated at once and the MS/MS spectrum could be deconvoluted for each selected ion (requires accuracy of <10 ppm). This will be tried with online separations in the near future.

Marvin Vestal (PE BioSystems) described his continuing efforts in MALDI-MS instrument design. The attributes he is aiming for include sensitivity, specificity (resolution, mass accuracy, selective ionization), speed, accuracy, dynamic range, and mass range. The sensitivity will always be limited by chemical noise, but the aim is to reduce the limitations of ionization and data handling. Vestal would like to achieve a sensitivity of 1 fmol with a data acquisition rate of 1 spectrum/s. The instrument he is designing to achieve these aims is a MALDI-TOF/TOF-MS. This system has an ion gate (with 500 resolution and no loss of sensitivity) after the collision cell so that metastable ions created after reacceleration are removed. Although this system is in the early stages of development, the data shown demonstrate that this instrument is meeting most of the stated objectives.

The hybrid quadrupole TOF (Qq-TOF) mass spectrometer developed a few years ago (9), which has now been commercialized, utilizes an ESI for ionization (10). Both Ken Standing (Univ. of Manitoba) and Brian Chait (Rockefeller Univ.) described the use of a MALDI ion source for introduction of ions into a modified commercial Qq-TOF, thus taking advantage of both the high-efficiency ion production of the MALDI and the ion isolation/fragmentation of the quadrupole system with a TOF mass analyzer. Standing presented data showing sensitivity of purified standards (e.g., Substance P) in the 70 amol range (1-min acquisition) for MS and 7 fmol for MS/MS with 10,000 resolution. This instrument offers similar advantages to the TOF/TOF described above.

Online MS analysis of capillary electrophoretic or chromatographic separations of peptide (or proteins) is most often achieved using ESI-MS. Barry Karger (Barnett Institute) described how very small quantities of peptides/proteins could be separated and analyzed using vacuum deposition onto Mylar audio tape for subsequent coupled MALDI-MS analysis. The approach had so far been multiplexed with the effluent of 12 capillaries being deposited under vacuum onto the tape. The approach is designed for high-throughput separations and mass analysis.

Proteomic analyses often employ 2-DE, but David Lubman (Univ. of Michigan) described a liquid-phase 2-D separation of proteins utilizing a novel MS. The requirements of his mass spectrometer were high sensitivity, low duty cycle, and fast response. He designed and built an ion trap to capture ions from the CE coupled to TOF-MS. The 2-D liquid-phase separation consisted of nonporous silica bead RP-HPLC (which provided good resolution <50 kDa) that was conducted at high pH followed by CE and MS. Whole cell lysates were analyzed with this system, and some of the data obtained were presented.

Biological applications. Brian Chait (Rockefeller Univ.) presented the culmination of an enormous amount of work at both the protein chemistry (mass spectrometry) and cell biology levels. The nuclear pore complex (NPC) in yeast is a massive structure (1,000 Å across with 8-fold symmetry) that regulates protein transport in/out of the nucleus. The first step in understanding this structure was to purify the complex and then identify every protein present. The protein fraction was separated by several different chromatographic steps followed by SDS-PAGE from which every visible band was excised and analyzed by MALDI-IT-MS. This was an especially daunting task as the NPC when isolated contains a snapshot of the proteins transiting the NPC at that point in time. Hence, of the 174 proteins identified, 29 were nucleoporins and only 14 were shown to be present in the NPC. These 14 proteins were characterized as being present in the NPC by a variety of techniques. Protein A (4.5 repeats of the Fc binding region) fusions with the proteins of interest were generated, and immunohistochemistry was performed on cells transfected with these constructs. Electron microscopy of hundreds of NPCs following transfection allowed stoichiometry and symmetry (nuclear/cytoplasmic/asymmetric) to be determined. Subcellular fractionation and high-pH extractions were also performed to further characterize localization biochemically. This elegant study has allowed a testable model for nuclear transport to be constructed.

Two examples of the utility of analysis of unfractionated or partially fractionated complex protein mixture digests (see next section) were presented by Scott Patterson (Amgen Inc.). As a first step in the understanding of the interchromatin granule clusters (IGC), a nuclear organelle which is a major site of mRNA splicing. Samples enriched in this structure were digested with trypsin, and the complex mixture of peptides was analyzed by data-dependent LC-MS/MS (8). Some proteins known to be present in these structures were identified together with 19 novel genes (including ESTs): Three of the genes were confirmed to be present in the IGC by immunohistochemistry of cells transfected with yellow fluorescent protein (YFP)-fusion constructs with counter staining of splicing factors. The other study presented identified 108 proteins present in a protein fraction obtained from isolated mitochondria treated with atractyloside [mimicking in vitro the permeability transition pore complex (PTPC) which occurs during apoptosis] (13).

Analysis of immunoprecipitates using a new affinity strategy was presented by Gitte Neubauer (EMBL). The new strategy is referred to as tandem affinity purification (TAP) and was developed by colleagues at EMBL (15). The system utilizes a double tag for higher

specificity and much reduced background. The human spliceosome immunoprecipitated under normal conditions (see Ref. 5 for same approach with yeast tri-snRNP) and using the TAP method were compared, demonstrating the utility of this approach.

The common theme of all of these applications is that MS was utilized early on to provide rapid and accurate protein identifications. The genes identified could then be further analyzed to attempt to determine their function.

The use of MS to identify proteins from 2-DE gels was also described by Al Burlingame (Univ. of California, San Francisco) and Reid Townsend (Oxford GlycoSciences). Burlingame described their work to identify protein targets of acetaminophen during acute toxicity and the intricacies of such analyses (14). Townsend described an Oxford GlycoSciences and Pfizer collaboration to separate by 2-DE and identify proteins from cerebrospinal fluid (CSF) in a study aimed at identifying markers for Alzheimer's disease. CSF is a compartment isolated by the blood-brain barrier but it is not just a filtrate of blood. It is produced by the choroid plexus and has a total central nervous system volume of about 90–150 ml that is turned over a few times per day. Comparative analysis of matched plasma CSF samples (in addition the normal/diseased samples) revealed that key plasma proteins (e.g., albumin, transferrin, IgG) showed markedly different relative ratios between plasma and CSF. For effective 2-DE analysis of these samples, a selective removal of albumin, IgG, transferrin, and haptoglobin had to be developed. This was accomplished by affinity depletion. Interestingly, many features in a 2-DE separation are albumin fragments (in fact, 4% of total features). Their study included 512 samples from 228 patients and resulted in 1,131 features (spots) being annotated. Potential markers of Alzheimer's disease were said to be identified.

Separate from the MS identification issues covered in most of the meeting, Kerstin Strupat (Univ. of Muenster) presented her work on MS analysis of noncovalent complexes. Here the challenge is to transfer noncovalent interactions that occur in the condensed phase to the gas phase. ESI-MS has been shown by a number of groups to work, but MALDI-MS analysis has proved more difficult. Examples of MALDI-MS analysis of noncovalent protein:protein (streptavidin tetramer and the macrophage migration inhibitory factor related proteins MRP-8 and MRP-14) and protein:ligand (aldose reductase:NADP) interactions were presented. Interestingly, analysis of the first laser pulse during a MALDI-MS analysis often allows investigation of noncovalent interactions that are not observed during subsequent pulses (16).

Analysis of complex protein mixtures without gel electrophoresis. The first stage of many proteome projects is the identification of the components comprising the system under study. This is of course the first step in understanding any biological system. As described above, an increasing (but still limited) number of laboratories have access to robotic systems requisite for the analysis of large numbers of spots from 2-DE. However, a trend in the field is emerging toward the elimination of the high-resolution protein separation step prior to protein identification by MS. In this approach, the entire enriched protein fraction is enzymatically digested (usually with trypsin), and the resulting complex peptide mixture is subjected to data-dependent LC-MS/MS. In this approach the peptides are separated by both hydrophobicity (RP-HPLC) and charge (m/z in the mass spectrometer) prior to ion selection by the MS control software (hence, data dependent). At this meeting, presentations from five groups demonstrated the utility of the approach to identify components of complex mixtures.

Analysis of immunoprecipitated proteins or enriched protein fractions (e.g., Golgi complex) by either gel electrophoresis followed by in-gel digestion and MS or digestion of the entire protein fraction and analysis by data-dependent LC-MS/MS using a Qq-TOF was described by Jyoyti Choudhary (Glaxo Wellcome). Batched MS/MS spectra were searched using the Mascot program (**http://www.matrixscience.com**). Data presented showed that if the immunoprecipitate was clean, then direct digestion of the mixture proved slightly more successful than analysis of gel-separated proteins. When an enriched Golgi complex from rat liver was separated by either 2-DE (135 spots) or 1-DE (77 bands) and in-gel digested followed by LC-MS/MS, significantly more proteins were identified from the 1-DE separation.

David Arnott (Genentech) described the proteomics component of Genentech's Secreted Protein Discovery Initiative, which also includes genomic, signal trap, expression, and functional analysis. Arnott evaluated three methods to identify proteins secreted from human umbilical microvascular endothelial cells (HUMECs) into 60 ml of serum-free media; 2-DE and 1-DE (with/without staining) followed by in-gel digestion, and direct digestion of the entire protein mixture. Digests were analyzed using the microcapillary system described above. Interestingly, direct digestion followed by data-dependent LC-MS/MS identified the most proteins, but all three methods were complementary in their hands (21 proteins identified by all three methods but no completely novel gene products).

Analysis of serum fractionated using the Cohn pH/ethanol precipitation protocol followed by digestion of the entire fraction prior to data-dependent LC-MS/MS was described by Karl Clauser (Millennium Pharmaceuticals) in the context of the studies of differences between wild type and ApoE −/− mice. Clauser also presented the bioinformatics flow for data handling, which utilizes a variant of the publicly available MS-Tag (**http://prospector.ucsf.edu/**) for protein identification and a de novo sequence interpretation program referred to as SHERENGA (2). Their stated aim is for searching to keep up with the LC-MS/MS. They have also been experimenting with the IEX ion-exchange protocol developed by Andy Link (7) as a means of decreasing the complexity of the sample and reducing the number of singly charged and highly charged ions as these are less likely to be identified. In one IEX

fraction, 87 plasma proteins were identified in a single run compared with 66 from an unfractionated sample.

Scott Patterson (Amgen) described Amgen's proteomics efforts, now in the third year. They are employing data-dependent LC-MS/MS of complex protein mixture digests. The stated aim is to reduce the complexity such that in an ideal situation only one peptide for each protein in the mixture is fragmented during LC-MS/MS. To achieve this aim, various affinity methods can be employed, and the use of cysteinyl peptide capture using either thiopropyl Sepharose or a biotin alkylating reagent, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide (HPDP-biotin), was described (13). The former was used in a large-scale analysis of urinary proteins where digestions of the unfractionated starting material, albumin/IgG depleted, or cysteinyl peptide captured or noncaptured were analyzed. The samples were analyzed with replicate LC-MS/MS runs using narrow mass ranges for ion selection for each run, thereby increasing the number of unique spectra selected for fragmentation. This analysis resulted in the identification of over 200 proteins, including a number of uncharacterized nucleotide sequences (e.g., ESTs). Smaller scale analyses are described above, in one case [soluble intermembrane proteins (SIMPs)] utilizing cysteinyl peptide capture to identify more proteins than with no fractionation. Data handling for this high-throughput effort was also briefly described. A number of the fractions being analyzed have some of the same components; therefore, to enhance the identification process, spectral matching of the database (>5 million spectra) is performed. This links identical spectra and therefore reduces the redundancy associated with re-searching already identified spectra.

Quantitative analysis of two samples without electrophoresis. MALDI-MS, using the surface enhanced laser desorption ionization (SELDI) surface, to search for disease markers in biological fluids was presented by Scot Weinbeger (Ciphergen Biosystems). In this approach, defined chemical/biochemical surfaces are utilized to allow fractionation of proteins from biological fluids in a reproducible manner. This reproducibility allows comparisons between different samples to be made. Weinberger described the search for markers of benign prostatic hyperplasia that, like prostate cancer, displays elevated prostate specific antigen (PSA) levels. The fraction exhibiting a difference between these samples was able to be enzymatically digested, and a number of peptides were generated. These were able to be fragmented using the MALDI Qq-TOF of Standing, described above. It appears as though there is a difference in the relative level of a seminogelin fragment between these two diseases, providing a potential differential marker. The method is sensitive but apparently limited to analysis of proteins less than about 20 kDa (a range not well characterized by 2-DE).

A combination gel/MS approach referred to as a "virtual 2-D gel" was presented by Phil Andrews (Univ. of Michigan). In this approach, proteins are separated by charge using thin-layer isoelectric focusing (IEF), and this gel is then subjected to MALDI-MS. By rastering through the entire IEF gel, a composite display of all acquired MALDI-MS spectra can be generated (hence, the virtual 2-DE). Such analyses would provide very accurate mass measurements, greatly assisting in posttranslational modification analyses as well as potentially quantitation.

Karl Clauser (Millennium Pharmaceuticals) described their efforts at utilizing already existing LC-MS/MS data to attempt to gain some quantitative/qualitative information as to differences between samples. Differences in serum protein levels between wild-type and ApoE −/− mice have been examined using this approach, which compares the MS ion current from peptides identified between LC-MS/MS runs of each sample. Comparison between runs is a difficult task, but data suggested that there is sufficient confidence to state a significant difference if there is a difference of a factor of 3 between some components of the samples.

An LC-MS/MS-based system was described by Steve Gygi (Univ. of Washington) for quantitative analysis of complex mixtures. The technology is referred to as isotope-coded affinity tag (ICAT) (6). The ICAT reagent described here is composed of three units: an affinity reagent (biotin), a linker region (one of two forms), and a reactive group (a thiol-specific reagent, iodoacetic acid). The linker region encodes the mass difference, with the light version having 8 hydrogens and the heavy version having 8 deuteriums. Thus the mass difference is 8 mass units (doubly charged ions will have an *m/z* difference of 4). Following reduction and alkylation of each of the two protein samples with one of the two reagents, the two samples can then be mixed together. All subsequent manipulations are performed as a mixture, culminating in tryptic digestion of the complex sample and capture of the cysteinyl peptides on avidin. The bound peptides are released and analyzed by LC-MS/MS, revealing paired signals of peptides. Calculation of areas under the peak for each paired ion from the LC-MS data provides an accurate record of the relative quantities of the proteins from each starting sample. The MS/MS spectra allow identification of the peptides. The approach was elegantly demonstrated with yeast grown on either galactose-containing media or ethanol-containing media. Proteins expected to be differentially regulated were observed, and, highlighting the advantages of analysis at the protein level as opposed to the mRNA level (e.g., microarray), alcohol dehydrogenase-1 (ADH1) was found to be oppositely regulated (as expected) to ADH2, to which it is 95% homologous. This is a very promising approach for quantitative analysis of complex protein mixtures.

A number of interesting posters were also presented at the meeting, and some of the presenters were given the opportunity to "advertise" their posters. These dealt with the same range of subjects presented in the oral sessions.

Conclusion. The organizers Ruedi Aebersold and John Stults brought together an excellent program for this meeting, with essentially all major laboratories in

this field being represented. The field has grown enormously over the past few years, and advancements presented at this meeting indicate an optimistic view of the future for proteomics. This very successful meeting provided the 162 attendees with the state-of-the-art in mass spectrometry and proteomics.

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Mass spectrometry and proteomics

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Plasma C3d levels and ischemic heart disease in type II diabetes.

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OBJECTIVE--To test the hypothesis that the complement system may be activated in patients with type II diabetes and CAD. RESEARCH DESIGN AND METHODS--The plasma C3d concentration was measured in 106 type II diabetic patients and 25 nondiabetic control subjects. The patient group was subdivided according to AER, and the groups were adjusted for age, sex, and known duration of diabetes. For the assignment to a given subgroup, normoalbuminuria was defined as AER < 15 microns/min, microalbuminuria as AER 16-250 micrograms/min, and macroalbuminuria as AER > 250 micrograms/min. The presence or absence of coronary disease was assessed through clinical examination, ECG, and coronary angiography. An RIA system was used for measurement of urinary albumin levels, and the plasma C3d concentrations were measured by ELISA. RESULTS--Within each of the AER-defined subgroups, the plasma C3d levels were significantly higher in patients with IHD than in those without. Thus, in the normoalbuminuric group, plasma C3d levels were 16.3 AU/ml (95% CI 13.9-19) in patients with IHD vs. 11.6 AU/ml (95% CI 10.5-12.7) in those without ($P < 0.001$). The corresponding data for the microalbuminuric and macroalbuminuric groups were 21.8 (95% CI 18.1-26.3) vs. 13.6 (95% CI 12.3-15.1) and 31.6 (95% CI 24.9-40) vs. 17.5 (13.6-22.6) AU/ml ($P < 0.01$), respectively. Patients with IHD also had significantly higher plasma C3d levels than normal control subjects, regardless of AER subgroup. A multiple logistic regression analysis demonstrated an association between the plasma C3d concentration and IHD and AER. CONCLUSIONS--Activation of the complement system may play a role in the development of macrovascular disease in type II diabetes.

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